Am J Hum Genet 35:1081–1085, 1983

The William Allan Memorial Award Address: Cell Mixing and Its Sequelae

ELIZABETH F. NEUFELD[1]

To receive the William Allan Memorial Award is a very special honor for which I am deeply grateful. I shall try to recount here the ideas and events that led to the discoveries for which the Award has been given.

My interest in mucopolysaccharidoses grew out of my previous studies on enzymes of UDP-glucuronic acid synthesis and utilization, first from plant sources (in the laboratory of W. Z. Hassid, at the University of California, Berkeley) and later from animal tissues. When I heard of a genetic disease called Hurler syndrome, with overproduction, storage, and excretion of mucopolysaccharides, I thought that faulty regulation of UDP-glucose dyhydrogenase, a key enzyme in the synthesis of the uronic acid groups of mucopolysaccharides, might be the cause. Following the lead of Danes and Bearn (Rockefeller Institute), who had shown by histochemical techniques that mucopolysaccharide storage occurred in fibroblasts cultured from the skin of Hurler patients, I learned to grow fibroblasts. After obtaining a biopsy from a Hurler patient through the kindness of Rodney Howell, then at Johns Hopkins, I began to investigate the feedback regulation of UDP-glucose dehydrogenase.

After some months I knew that I was on the wrong track; the enzyme from the Hurler fibroblasts was regulated in a normal way. These exploratory experiments were not published, the underlying hypothesis was incorrect, but I had become hooked on the problem: What *was* the biochemical basis of Hurler syndrome? When Joseph Fratantoni joined me in the fall of 1967, we decided to test the prevailing doctrine of mucopolysaccharide overproduction. We examined the kinetics of incorporation of radioactive sulfate into various metabolic pools of mucopolysaccharide, observed very diffferent patterns for normal and patients' fibroblasts, and interpreted the difference to signify faulty degradation of mucopolysaccharide, rather than oversynthesis.

Later that year, we became intrigued by a new paper of Danes and Bearn, demonstrating that fibroblasts of heterozygotes for Hunter syndrome (an X-linked mucopolysaccharidosis) were a mixed population of normal and Hunter cells, in agreement with the Lyon hypothesis of X-chromosome inactivation. I had not known of this hypothesis, although it had been published 5 years earlier, and tried to guess its consequence to the heterozygotes. It seemed that with half their

---

Presented on the occasion of receiving the William Allan Memorial Award at the annual meeting of the American Society of Human Genetics, Detroit, September 29–October 2, 1982.

[1] Genetics and Biochemistry Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, Bethesda, MD 20205.

cells storing mucopolysaccharide, the heterozygotes should have an attenuated form of the disease. But a literature search and personal inquiry established that Hunter heterozygotes had a normal phenotype, and therefore it seemed necessary to postulate that the normal cells were somehow assisting the abnormal ones. As newcomers to the field, we had no cells of Hunter heterozygotes with which to test this idea, but we speculated that in light of the Lyon hypothesis, we could make a reasonable facsimile of a heterozygote by simply mixing normal and Hunter cells in a petri dish.

This experiment was not performed right away, but a few weeks later Joe Fratantoni reported that he had inadvertently mixed Hurler and Hunter cells while setting up a labeling experiment; in view of our discussion, he did not discard the mixture but completed the experiment. The results were dramatic—the mixture had an almost normal labeling pattern. We subsequently designed an experiment that included mixing Hurler, Hunter, and normal cells in various combinations. Again, interaction of cells of different genotype was clear: Hurler and Hunter cells corrected each other and both were corrected by normal cells.

Further work showed that cell-to-cell contact was not necessary, that the cross-correction could be obtained by use of conditioned medium. The cells were secreting "corrective factors" that we undertook to isolate and identify. At the first stages of purification, it became apparent that the factors behaved as proteins. We took as our working hypotheses that the corrective factors were degradative enzymes needed for the catabolism of mucopolysaccharide, that some of them might be enzymes which had not yet been described, and that absence of a unique and specific factor (i.e., enzyme) was the basis of each mucopolysaccharide storage disease.

There followed 2 very tedious years of factor purification followed by 2 exciting years of detective work to assign a function to each factor. My collaborators during those years, in the order in which they joined the project, were: Ulrich Wiesmann, who discovered heterogeneity within the Sanfilippo syndrome: Michael Cantz, who purified the factor corrective for Hunter cells; Robert Barton, who purified the factor corrective for Hurler cells; Hans Kresse, who purified the factor corrective for cells of Sanfilippo patients of the A subtype and showed it to be heparan N-sulfatase; and Gideon Bach, who identified the Hurler and Hunter factors as the enzymes α-L-iduronidase and iduronate sulfatase, respectively. The enzyme activity associated with each factor was the same as the activity missing in the cells for which the factor was corrective. Clara Hall, who had been associated with this work since its inception, found a deficiency of β-glucuronidase in a culture sent to us by William Sly of Washington University. In a reversal of the chronology followed for the other mucopolysaccharide storage disorders, the enzyme deficiency was discovered first and the enzyme (we used a commercial preparation of bovine β-glucuronidase) was then found to have corrective factor activity for the deficient cells.

By 1972, the structure of dermatan sulfate and heparan sulfate had been clarified by carbohydrate chemists, and the mucopolysaccharidoses could be ascribed to the deficiency of one of six enzymes required to hydrolyze specific linkages in the two polymers. Today, the mucopolysaccharidoses number 10 enzyme deficiency diseases, including four specific to heparan sulfate (Sanfilippo syndrome) and

two to keratan sulfate (Morquio syndrome). They include a deficiency of the only known lysosomal enzyme that is not a hydrolase but a transferase, requiring acetylCoA as co-factor (Sanfilippo C). Since there are still some known linkages without an associated enzyme deficiency, and perhaps some yet unknown linkages, the list will surely be extended in the future.

The elucidation of enzyme defects in mucopolysaccharidoses represents the combined work of many laboratories, including those of Albert Dorfman (University of Chicago), John O'Brien (University of California), Kurt von Figura and Hans Kresse (University of Münster), and Hayato Kihara and Arvan Fluharty (Lanterman State Hospital).

The discovery of correction in cultured fibroblasts kindled the hope for some analogous therapy for mucopolysaccharidoses patients. Enzyme replacement for storage diseases had been proposed in 1964 by the lysosomologists of Louvain—Hers, van Hoof, and others in the laboratory of DeDuve. But first we had to learn about the mechanism of enzyme uptake and about the basic biology of lysosomes and of the enzymes that reside in them.

Our first clue to the complexity of the correction process came from an experiment by Gideon Bach, designed to measure how much Hurler factor (i.e., α-L-iduronidase) entered into Hurler cells in the process of correction. The answer was unexpected: it could be as high as a third of the administered dose. Such remarkable uptake could not be ascribed to nonselective "fluid" endocytosis; we had to postulate some specific chemical structure on the enzyme that would allow it to bind to the cell surface and thereby enhance its uptake. Evidence for a binding step was later provided by Gloria Sando, from a careful study of the kinetics of uptake. Experiments by Scot Hickman and Larry Shapiro showed the specific structure, which we named a "recognition marker," to be distinct from the catalytic site of the enzyme and to be a carbohydrate. Its key feature was identified in 1977 as mannose-6-phosphate by Kaplan, Achord, and Sly (Washington University). The entire marker is, in fact, an aspargine-linked oligosaccharide with two *N*-acetylglucosamine and six or more mannose residues, one or two phosphate groups, and several possible isomeric forms.

The significance of the recognition marker in the transport of enzymes to lysosomes became evident from studies of fibroblasts of patients with I-cell disease. These cells are characterized by a pleiotropic defect of lysosomal enzymes, a number of enzymes being partially or profoundly reduced intracellularly but greatly elevated in the culture medium. In 1972, Scot Hickman and I showed that the enzymes secreted by I-cell fibroblasts were noncorrective, were not taken up by other cells, and thus, seemed to lack the recognition marker. Seven years later, Andrej Hasilik showed that several newly made I-cell enzymes lacked mannose-6-phosphate, and in 1981, Hasilik, Waheed, and von Figura (University of Münster) and Reitman, Varki, and Kornfeld (Washington University) identified the primary deficiency in I-cell disease to be the enzyme which transfers phospho-*N*-acetylglucosamine residues from UDP-*N*-acetylglucosamine to newly made enzymes destined for lysosomes.

As shown in many laboratories, lysosomal enzymes share early biosynthetic reactions with other glycoproteins: translation on membrane-bound polysomes, entry into the endoplasmic reticulum, glycosylation from a lipid intermediate,

and transport to the Golgi. Only there does their fate diverge from that of glycoproteins destined for other locales. Lysosomal enzymes acquire the recognition marker by attachment of phospho-*N*-acetylglucosamine to mannose followed by removal of the *N*-acetylglucosamine. Now equipped with the mannose-6-phosphate recognition marker, the enzymes are recognized by a specific receptor for transport to lysosomes.

Part of the lysosomal enzymes may be secreted, and can be endocytosed upon binding to a specific receptor on the cell surface. I am particularly fond of this pool of extracellular enzymes, for it is responsible for the phenomenon of cross-correction in culture and presumably for the normal phenotype of Hunter heterozygotes in vivo.

A receptor specific for lysosomal enzymes was first perceived as a necessary component of the selective uptake system. Direct evidence for the receptor was obtained by Leonard Rome through binding of α-L-iduronidase to the surface of human fibroblasts. Gary Sahagian and G. William Jourdian (University of Michigan) purified the receptor from bovine liver and showed its specificity for the mannose-6-phosphate recognition marker, and William Sly (Washington University) pointed out the role of the receptor in the intracellular transport of enzymes to lysosomes.

Once in lysosomes, the enzymes undergo further changes, which we have called "processing" or "maturation" or, perhaps more correctly, "aging." The protein chains may be shortened or nicked, the phosphate groups and some carbohydrate residues removed. The degradative events continue, and eventually the enzymes lose their catalytic activity. These changes are readily observed after pulse-chase labeling of fibroblasts with radioactive precursors, followed by isolation of the radioactive polypeptides by immunoprecipitation and electrophoresis, a technique introduced into our laboratory by Andrej Hasilik.

The lengthy pathway for making lysosomal enzymes leaves much room for genetic errors. I-cell disease has been mentioned; pseudo-Hurler polydystrophy is a milder variant with a partial deficiency of the same enzyme, phospho-*N*-acetylglucosamine transferase. Richard Proia reported at this meeting on an altered α-chain in a non-Jewish patient with late-infantile Tay-Sachs disease. This α-chain is glycosylated but neither phosphorylated nor secreted nor proteolytically processed. It is insoluble, requiring strong detergent for extraction. We speculate that the insoluble α-chain fails to progress from the endoplasmic reticulum to other organelles. Yet other mutants (e.g., glycosylation mutants, receptor mutants, post-receptor endocytosis mutants) have been isolated in CHO cells by April Robbins but have yet no human counterpart.

Such is the biology of lysosomal enzymes which we must understand in order to devise therapeutic strategies. Its complexity is even greater than I have indicated: mannose-6-phosphate is not a universal marker for endocytosis into all cells; direct cell-to-cell transfer may operate where pinocytosis does not; alternative pathways probably exist for the transport of endogenous enzymes to lysosomes.

Understandably, therapeutic attempts did not wait until all the biochemical details had been worked out. Starting over a decade ago with plasma infusions by DiFerrante (University of Texas), lymphocyte infusions, plasma exchange,

and transplantation of fibroblasts have been tried in the hope of reproducing in vivo the correction so readily observed in cell culture. Unfortunately, none of these procedures have provided lasting clinical benefit, either because the donor materials provided too little of the relevant enzymes and/or because the enzymes failed to reach their target. We watch with cautious optimism the bone marrow transplantations initiated in London by Hugh-Jones. Animal models are becoming available to test the efficacy of such procedures. Advances in molecular genetics provide new technology that will accelerate our studies and improve the prospects for novel kinds of therapy.

ACKNOWLEDGMENTS

I have named some of my associates in the text as I described their experiments; constraints of space did not permit me to mention the work of many others, and I ask their forbearance. I thank my colleagues in other institutions who have sent me biopsies, cells, substrates, and other essential materials. I am particularly grateful to the patients and their families, whose active collaboration made the work possible.